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(54) Title: VACCINE AGAINST ISA VIRUS

(57) Abstract: The invention relates to vaccine against infectious salmon anaemia (ISA) virus, DNA sequences endocing immunogenic proteins from ISA virus, diagnostic kit for detection of ISA specific nucleic acid sequences and proteins and exploitation of the ISA virus genome or parts of it as a general model organism.

Title

Vaccine against ISA virus

Field of the invention

The present invention concerns a vaccine against infectious salmon anaemia (ISA), nucleotide sequences encoding for immunogenic proteins from ISA virus, in addition to exploitation of the ISA genome or parts of it within biomedicine (model organism), in human medicine as well as in experimental use, and the use within preventive medicine in fish, hereunder diagnostic use for demonstration of ISA specific nucleic acids or proteins.

Background of the invention

Infectious salmon anaemia (ISA) is a virus disease limited to Atlantic salmon (Salmo salar L.) in cultivation. The disease was diagnosed first time in 1985 in parr (the Bremnes outbreak). The disease has only been found in Atlantic salmon in salt water or salt water added to fresh water. Administrational measures issued by the authorities, as stamping out (and isolation) of affected fish farms followed by disinfection and restrictions on trading/moving of fish in the area have limited the number of outbreaks from the peak around 1990, but in the later years, the disease has increased in extension. Primarily, ISA has been a Norwegian salmon production problem, and in 1998 15 outbreaks were registered. However, during the period 1997-98, ISA was found and verified in Canada (97) and Scotland (98).

The disease is caused by a general infection which among others cause severe anaemia and bleeding lesions. The disease spreads slowly in an infected fish farm, and the mortality can vary from 15-100%. There is no available cure against ISA. The goal of implemented control measures is to minimalise the risk for exposure of ISA virus to the salmon. The demonstration of infection implies stamping out of all fish in an affected fish farm and disinfection of the localities. There is no available vaccine.

Due to the severe economical strains the disease implies to society in general and to the individual fish farmer, a good and secure diagnostic procedure is of

importance. Diagnosis of ISA is still based on a combination of macroscopical and microscopical observations of dead/dying fish (pathological/histological investigations). Recently, researchers have had success in growing ISA virus in cell culture (1), which is very time- and resource demanding. An indirect immune fluorescence test for the demonstration of infective material has been developed for the use in tissue sections and tissue impressions (2). A quick-test, - a RT-PCR (reverse transcriptase polymerase chain reaction) –test to demonstrate ISA virus in salmon has been developed as well. It can also be used in ISA infected fish showing no clinical signs of disease (3). This test is ready for the use in the context of diagnosis and mass investigation.

The most likely preventive action against ISA is the development of a vaccine and other influences of the natural defence system in the salmon.

ISA virus contains a negatively charged single-stranded RNA genome of 8 segments. The total size of the segments is 14,5 Kb (1,5 x 103 base pairs). The virus replicates itself in the nucleus. It is a 100-120 nm enveloped virus with 10 nm peplomers, and it separates itself from the cell membrane by budding. The entrance of ISA virus in cells is pH-dependant. ISA virus harbours hemagglutinating and hemadsorbent abilities (3). All listed features indicate that the virus belongs to the family Orthomyxoviridae, implying an influenza-like virus.

The immune system of salmon share many similar properties with the immune system of mammals. Accordingly, it is possible to draw a number of parallels. Teleosts posses immunocompetent cells such as B- and T- lymphocytes, lymphokines, complement factors and they produce immunoglobulines. Farmed salmon is vaccinated against important bacterial infections. In Norway, vaccines against the IPN virus is also available, but the effect of these vaccines is discussed. The demand for new and more effective vaccines against virus diseases in farmed fish is substantial. DNA-vaccine is an important candidate among vaccine strategies to choose and has been described in various contexts (4). At DNA immunisation against for instance flue virus, protective effects not only directed against the actual antigen variant of the virus used in the vaccine, but also effect against antigenically different virus have been observed (5). This broad immune response can possibly be

explained by a good cellular response. It is assumable that a good cellular immune response provides a far better protection against ISA than humoral immune response alone. This is due to the fact that the cellular immune response is directed against a broader range of antigens, and the cellular response is longer-lasting than the humoral response.

The interferon system is also an interesting part of the teleost immune system against influenza-like viruses including ISAV. The interferons induce retardation of virus replication and are of particular importance before the establishment of a specific immune response. Interferon-induced proteins, known as Mx-proteins, are important in the retardation process of influenza-like viruses. For instance, mice lacking functional Mx-genes do not survive influenza infections (6). Mx-genes are also demonstrated in salmon (7), but these do not seem to repress ISA virus sufficiently to prevent disease. The ISA virus has possibly adopted to salmon to such a degree that it may replicate despite the Mx-response of the host. Mx-proteins from human as well as mouse appear to restrict the replication of ISA virus in cell cultures (8).

There is a relatively large degree of homology between Mx-genes from mammals, birds and fish, indicating the severe threat of influenza-like viruses to the species, creating a selection pressure to the benefit of individuals carrying Mx-genes. It is therefore assumable that influenza-like viruses have existed in the marine environment over a substantial period of time. Farming of salmonids in sea-water has established conditions for an effective cultivation and distribution of virus, disease outbreaks represent reminders of the existence of influenza-like viruses in the marine environment. A virus reservoir in the marine environment has not yet been identified, thus complicating preventive measures.

Electron microscope studies have demonstrated that ISA virus buds from endothelial cells in blood vessels in several different organs (9). Following experimental challenge tests, virus particles have been identified in most organs, making the disease different from influenza infections in humans where infection usually is limited to the respiratory system. Orthomyxovirus possess 3-4 different surface proteins,; hemagglutinin is regarded to be of particular importance, being

responsible for choice of host cell, this due to receptor recognition and thus the binding to the host cell. Hemagglutinin is likely to harbour similar abilities in the ISA virus. Host-cell restricted and surface located protein splitting enzymes (proteases) are necessary to activate the hemagglutinin, making the transport of virus into the cell possible. In this context, the accessibility and tissue distribution of suitable proteases in addition to the accessibility of cellular surface molecules which can act as receptors for ISA virus is of importance. The wide-spread tissue distribution of ISA virus during infection indicates that if the infectibility is dependant on proteolytic activation of virus proteins, this activation is conducted by ordinarily existing proteases. This can partly explain the pathogenicity of the ISA virus which may give up to 100% mortality in certain outbreaks.

Previously, procedures for immunisation of aquatic species by DNA expression systems have been described. See European Patent application no. 839913/964713 (NO). Herein is described the procedure of immunisation using DNA vaccines directed against various aquatic viruses, ISA virus is not described but mentioned in the Norwegian application in claim 11 page 39. Any specific references concerning ISA virus are not mentioned, neither with respect to which gene sequences which may be efficient, nor methods to sequence them.

The difference between human and fish vaccine is limited. Presently, no DNA vaccine is available commercially. The principle is the same, but the application will be different, and of course infective agent. A limited number of vaccines against virus diseases are available in aquaculture production. DNA vaccines represent a new and promising approach in this context. DNA-vaccination implicates administration of antigen-expression vectors which give protein synthesis in situ in tissues in the vaccinated animal. DNA vaccines have experimentally been shown to give protection against influenza virus in mice (close relative to ISA virus) (10, 11, 12, 13). In contrast to recombinant or subunit vaccines, DNA vaccines will mimic attenuated or living, recombinant vaccines due to their possibility to initiate the production of cytotoxic T-cell responses and antibody responses which recognise authentic conformation dependant epitopes. The matrix proteins in orthomyxovirus is by number the predominant protein in the virus particle and has been demonstrated to be

of importance to give cross protection (e.g. protection against different strains of influenza-virus which would give reduced protection due to antigen/genetic drift if this was not the case) in mice (14). The matrix protein should therefore be a part of a DNA-vaccine which should protect against ISA virus (5,10).

The traditional fish vaccines are injected intraperitoneally, and an admixture of adjuvance to increase the effect is used. Oil mixtures based on animals/vegetables are mainly used, which may cause severe side effects in the context of peritonitis which may lead to fusions and reduced appetite. DNA vaccines do not demand adjuvance of this kind to be effective. In certain cases, the use of liposomes may increase the effect, but a good response following intracutaneous and intramuscular injections without admixtures is expected. It is also planned to investigate if sufficient effect after dip- or bath vaccination is raised.

More detailed description of the invention

The present invention relates to a vaccine against ISA virus, characterized in that it embraces cDNA sequences which are complementary to the ISA virus RNA genome and which encode for immunogenic proteins from the virus, DNA sequences which encode at least one matrix protein from the virus and/or DNA sequences which encode matrix protein and/or protein integrated in the virus membrane. The invention also includes DNA sequences which are characterized in that they encode immunogenic proteins from ISA virus, at least one matrix protein from ISA virus, matrix protein and/or another protein integrated in the ISA virus membrane and/or a DNA sequence, characterized in being represented by nucleotide sequences shown in SEKV. ID NR.:1, sequences of relatively high degree of similarity to this in addition to parts of it.

A DNA vaccine has been experimentally produced and some vaccine experiments have been carried out. The results indicate that the prototype is promising and has a certain protective effect, but an optimalization is still needed.

The invention also includes a vector which contains said DNA sequence, procedure for production of this DNA, the use of this DNA sequence in diagnostic detection of ISA virus specific proteins/nucleic acids, and detection of antibodies

directed against immunogenic ISA virus proteins – encoded by the said DNA sequence.

Fish possess an immune system which is relatively similar to what is known from mammals. In salmon, which have survived ISA infection, ISA virus specific antibodies can be detected. The applicant has cloned and sequenced various parts belonging to the ISA virus, encoding virus proteins important for stimulation of the salmon immune system for eventually providing a protective response against ISA. Based on this and in accordance with the invention, a vaccine is produced. The vaccine contains DNA (optimal and determined construction) which provides a protection against ISA disease when administered to salmon. For instance, following injection, cells will engulf DNA from the vaccine and express virus proteins. This induces an immune response which is similar to what happens following a natural infection and thus provides a better protection compared to the response which only is based on inactivated/killed virus or recombinant proteins. The reason for this is that the proteins which are expressed by a DNA vaccine will be processed as cellular proteins and be presented on the cell surface in the context of antigen-presenting molecules, as proteins from intracellular parasites such as viruses.

In addition a RT-PCR-test has been developed for the use in ILA-virus detection in organ material from salmon. In this test primers reacting with ILA-virus sequences, as described in this invention, have been used. Results from all organ material so far tested indicate accordance to other similar test. Such test is meant for use in mass screening in connection with surveillance, moving fishes from one location to another and disease outbreaks.

The DNA sequence which is shown in SEKV. ID NR.:1 encodes a polypeptide of 391 amino acids. The molecule weight of this protein and its encoding gene segment is analogues to what is the case for the matrix protein of the influenza virus. In influenza virus, the most numerous protein in the virus particle is the matrix protein which is present as a non-integrated membrane protein located beneath the virus membrane and provides its rigidity and stability.

Brief description of the drawings

Figure 1. The graphs illustrate the results of vaccination trial using the ISA-klon1-pEGFP-N1 plasmid as the vaccine (Example 10). Two parallel tanks were used, 50 salmon of approximately 25 grams of size, 25 salmon in each tank were injected twice with ISA-klon1-pEGFP-N1 plasmid while 25 salmon were injected twice with a control plasmid, pEGFP-N1 The horizontal axis denotes the number of days after challenge with virulent ISA virus. The vertical axis denotes the number of accumulated dead salmon. In tank A no difference between the control and vaccinated groups was observed, while in tank B a statistically significant difference was found between the control and vaccinated groups.

Figure 2. The graphs illustrate the results of vaccination trial using the ISA-klon1-pEGFP-N1 plasmid as the vaccine (Example 10). The graph is the same as Figure 1, but it only illustrates the results observed in tank B. A statistically significant difference was found between the control and vaccinated groups. Compared to tank A there was a delay in tank B before the control injected fish began to die. This could indicate that a prolonged period of time between vaccination and exposure is better.

Example 1. Isolation of the gene segments

Cells were harvested from a 175 cm^2 cell culture flask with SHK-1 cells which had been infected with ISA virus (Glesvær strain) for 5 days. Cells were washed twice in PBS. mRNA was subsequently extracted with oligo-dT cellulose in accordance with the producer's instructions (mRNA purification kit, Pharmacia, Uppsala, Sweden). mRNA was precipitated with ethanol and diluted in diethyl pyrocarbonate (DEPC) treated water and concentration of mRNA was determined by measuring OD_{260}. Totally 1.0 µg mRNA was subsequently used for first strand synthesis by reverse transcription in accordance with the producer's instructions (TimeSaver cDNA Synthesis Kit, Pharmacia). The primer used for this reaction was an oligo-dT primer with a Not I restriction site in the 5'-end. Following the second strand synthesis, a EcoR I adaptor was attached to cDNA using T4 DNA ligase at 16^0 C for 2 hours. This cDNA was subsequently cut using Not I and put into a EcoR

I/Not I cut pCRII plasmid. The new plasmid containing the insertion was used for the transfection of E. coli. Plasmids from transformed bacteria were purified by mini-prep procedure and cut by EcoR I/Not I and separated by electrophoresis in an agarose gel. Plasmid insertions were isolated from the gel and purified (Genclean, Bio 101, Vista, CA, USA) and stored at -20^0C before being used in hybridization reactions.

In the hybridization reactions, total RNA was extracted from ISA infected and non-infected SHK-1 cells. RNA was extracted by using 8.5 ml TRIzol solution (Gibco BRL, Gaithersburg, MD, USA) per 175 cm^2 cell culture flask following removal of medium and washing of the cells with PBS. This suspension was subsequently removed from the cell culture flask and transferred to a sentrifuge tube. 8.5 ml chloroform was added and sentrifuged for 50 min. at 3,600 x g. The water layer was removed and RNA was pelleted by adding 0.7 volume units of isopropanol. The RNA pellet was washed in DEPC-treated 70% ethanol and diluted in 0.5 ml DEPC-H_2O. RNA was subsequently denaturated using 1 M glyoxal, 10 mM $NaPO_4$ at 50^0C for 1 hour, and 10 µg denaturated RNA was used in a 1% agarose gel containing 10 mM $NaPO_4$ as buffer. RNA was subsequently blotted to a nylon membrane (Hybond N+, Amersham, Buckinghamshire, UK) and fixed for 2 hours at 80^0C. Hybridiztion was carried out by ^{32}P probe labelling (Rediprime DNA labelling system, Amersham). cDNA from ISA virus infected cell cultures was used as probes. The hybridizations were carried out at 50^0C over night and washed in 2 x SSC, 0.1% SDS at room temperature in 2 x 5 min followed by 0.1 x SSC, 0.1 % SDS at 68^0C for 2 x 15 min. Probes which gave positive signals from ISAV-infected SHK-1-cells and no signals from non-infected cells were tested for ISA virus specificity with Southern blot hybridization, where measure was total-DNA from non-infected SHK-1 cells cut with either *EcoR I*, *BamH1* or *Hind III*. These probes were also tested in hybridization reactions in which RNA was extracted from pelleted ISA virus from cell culture media and from ISA infected SHK-1 cells. Previous to pelletation of virus from cell culture supernatant, the medium was sentrifuged at 3,000 x g for 30 min and subsequently ISA virus was pelletated at 100,000 x g for 3 h. RNA from this

pellet was divided in 3 fractions; fraction one was treated with RNaseA, fraction two with RNase free DNase and fraction three remained untreated.

One clone gave positive signals in the hybridization reactions with total RNA from ISA infected SHK-1 cells, and no signals from neither RNA nor DNA from non-infected SHK-1 cells. When pelleted ISA virus was used as target, positive signals were obtained against a gene segment of ca. 1.3 kb. This signal vanished subsequent to treatment of ISA virus pellet with RNaseA, however, treatment with RNase free DNase had no effect.

The present ISA virus specific gene sequence was sequenced in an automatic DNA sequencer, DNA-sequencer (ABI Prism 377, Perkin Elmer Applied Biosystems, Foster City, CA, USA). The nucleotide sequence is shown in SEQ ID. NO.:1.

ISA virus specific gene segments were subsequently further verified as ISA virus specific by determining the terminal 5' and 3' ends by RACE (rapid amplification and cDNAends). In influenza virus, these 5' and 3' terminal ends are constant between the different segments and also partly complementary between themselves. With respect to the sequences according to present invention, these conserved 5'and 3'ends were identified, this serving as a further verification of the ISA virus specificity.

Example 2. Production of DNA-constructs for the expression in eucaryot cells.

Based on the present sequence for gene segment 7, an open reading frame consisting of 1173 nucleotides is identified. This reading frame will theoretically encode a protein of 391 amino acids with a total molecular weigh of ca. 42.8 kD.

To express the large open reading frame (assumed matrix protein gene) in eucaryotic cells, this gene sequence was cloned into the pEGFP-N1 vector (Clonetech. GenBank accession number U55762). In this vector, the expression of the cloned gene is controlled by a cytomegalovirus promotor, which has been demonstrated to be effective in fish. The expressed protein is a fusion protein between GFP (green fluorescent protein), this is located to the N terminal of the protein, and is therefore expressed only if the pre-encoded protein also is expressed.

In short, this is achieved by the following method: Two PCR primers were constructed complementary to each side of the open reading frame, and in addition, each possessed a restriction enzyme site in the 5' terminal (*NaeI* and *kph*). The restriction enzymes were chosen from these criteria: a) no restriction sites in the actual gene sequence, b) the restriction site is present in the expression vector.

The PCR primers which were used for amplification of the mentioned open reading frame for subsequent cloning into the pEGFP-N1 vector are shown beneath:

PRIMER: 5`-GG-GCT-AGC-**ATG**-GCA-CGA-TTC-ATA-ATT-TTA-3`
NAME: KLON1-EGFP-F1
POSISTION: 7-28, KLON1.
DIVERSE: Starting codon in bold letters. *NheI* site is underlined.

PRIMER: 5`-G-GGG-TAC-CGT-**AGC**-AAC-AGA-CAG-GCT-CGA-TGG-3`
NAME: KLON1-EGFP-R1
POSITION: 1179-1159, KLON1.
DIVERSE: Last codon (that is inverse of this codon) previous to stop codon is in bold letters. *KpnI* site is underlined. Two nt (GT) were put in between last codon and *KpnI* to obtain correct reading frame for the GFP protein in the pEGFP-N1 vector.

Following PCR was performed:

26 µl H_2O
5 µl 10X Taq polymerase buffer without MgCl2
8 µl 1.25 mM dNTP
1.5 µl MgCl2
1 µl W-1
1.5 µl KLON1 EGFP-F1 (15 pmol/µl)
1.5 µl KLON1 EGFP-R1(15 pmol/µl)
0.5 µl Taq-polymerase
45 µl totally

Subsequently, 5 µl cDNA was produced from RNA which was extracted from organ material from salmon experimentally infected with ISA virus, the Glesvær strain.

Following PCR was performed:

Initially 5 min at 94°C.

Thereafter 35 cycles with:

30 sec 94 °C

1 min 55 °C

30 sec 72 °C

Thereafter 7 min at 72 °C, and 4 ° indefinitely.

10 µl of the PCR solution was used for electrophoresis in a 2 % agarose gel and subsequently stained with ethidium bromide. DNA fragments with correct size according to the open reading frame were cut out from the gel and purified with Geneclean.

DNA from the bit of gel was diluted in 10 µl H_2O.

The DNA was subsequently cut by *NheI/KpnI*

10 µl DNA from KLON1-EGFP-N1/KLON1-EGFP-R1PCR

2 µl React 3 (GIBCO buffer for restriction enzymes)

5 µl H_2O

1 µl *NheI*

1 µl *Kpn I*

20µl

Incubated at 37° C in 2 h.

1 µl pEGFP-N1

2 µl React 3 (GIBCO buffer for restriction enzymes)

15 µl H_2O

1 µl *NheI*

1 µl *Kpn I*

20µl

Incubated at 37° C for 2 h.

Everything was run in electrophoresis in 2% agarose gel and subsequently stained with ethidium bromide. The DNA fragments were cut out and isolated and DNA was extracted (Geneclean). DNA from each of the bits of gel was subsequently diluted in 5 µl H_2O. 1 µl of this was used to measure the DNA amount (OD_{260}). Relative mass relation ratio between *NheI/Kpn I*-cut pEGFP-N1 and the PCR amplified *NheI/Kpn I*-cut DNA-segment was calculated.

Ligation reaction: The mass relation ratio between *NheI/Kpn I* I cut pEGFP-N1 and the PCR amplified, *NheI/Kpn I* cut DNA segment was in the area of 1:1, 1:3.

2 µl *NheI/Kpn I* I cut pEGFP-N1
2 µl *NheI/Kpn I* cut DNA segment
1 µl 10 x ligase buffer
4 µl H_2O
1 µl T4 DNA-ligase.
10 µl

Incubated at 16°C for 4 h.

Fresh, competent *E.coli* cells were transfected in accordance to Maniatis(15).

50 µl and 200 µl from each tube were cultivated on agar plates containing Kanamycin and incubated overnight at 37°C.

5 colonies from these agar plates were used for miniprep of plasmids (Qiagen miniprep). The plasmid pellet was diluted in 20 µl H_2O and used in the restriction cutting *NheI/Kpn I* as this is described previously.

A bacterium clone, which contained plasmid with the correct fragment (ISA klon1-pEGFP-N1) (according to size at electrophoresis), was used for subsequent expression. As backup, 100 µl bacteria solution from the miniprep was spread on agar plates containing Kanamycin, incubated overnight, and the bacterium colonies were diluted in 1 ml LB medium containing 15% glycerol and put in storage at – 70^0C.

There are also shorter, potential reading frames (see sequence fig.). Influenza virus is the only virus where RNA splicing has been demonstrated, and therefore, similar abilities can not be ruled out in the ISA virus.

Example 3. Amplification of DNA sequences

The mentioned DNA sequence which is complementary to RNA from gene segment 7 in the ISA virus was amplificated in following ways:

1) PCR using primers complementary to the 5' and 3' terminals of the mentioned sequence. CPR-amplification was used previous to cloning into a vector as described in example 1 and 2, target RNA was then total RNA from an organ from experimentally ISA infected fish, such that possibilities for eventual artificial mutation should remain as little as possible. The PCR requirements used are described in example 2.

2) Amplification of DNA from ISA virus gene segment 7 sequence in the pCRII vector. This sequence lacked the extreme 5' and 3' terminals found using the RACE method (described in example 1). 25 ng of this plasmid (ISA virus gene segment 7-pCRII) was transfected into competent E.coli cells (TOP10'). E.coli which had been transfected were selected on the basis of resistance against Kanamycin, which is an ability which is encoded by pCRII. Bacterium colonies were tested for plasmid transfection by the aim of miniprep K (Qiagen miniprep). One colony containing the right insertion was cultivated in 5 ml LB medium containing Kanamycin, and the plasmid was purified (Qiagen miniprep). This plasmid was used as DNA source for nucleotide sequencing, which was carried out using an automatic DNA sequencer (as described in example 1).

3) Amplification of DNA which consists of open reading frame insertion in pEGFP-N1 (ILA-klon1-pEGFP-N1).

25 ng of (ILA-klon1-pEGFP-N1) was transfected in E.coli. Colonies were selected based on Kanamycin resistance. Thereafter testing for content of correct insertion was performed by miniprep (Qigaen miniprep) and restriction enzyme analysis (NheI/KpnI cutting as described in example 2). A colony of E.coli containing plasmid with correct insertion was used for amplification of ILA-klon1-pEGFP-N1. Qiagen Maxiprep and Gigaprep were used for this purpose according to manufacturer's instructions.

Example 4. Expression in a cell line

The ILA-klon1-pEGFP-N1 plasmid was transfected into BF-2 cells. BF-2 cells represent a standard fish cell line. These were cultivated in 96-well plates following standard procedures. FuGene (Boehringer-Mannheim) transfection medium was used. 1,4 µg DNA was sufficient for 25 wells. Totally 4 µg FuGene/DNA solution was added to each well. The wells contained medium and the cells were not washed or treated in any other way in the process of transfection. The success of transfection was measured by examining the transfected cells in a UV light examination microscope. UV light positive cells would indicate production of GFP (green fluorescent protein), the reading frame for this protein is located downstream of the cloned, open reading frame of the ISA virus segment 7, and will only be expressed in the case of expression of this protein. Expected molecular weight of this fusion protein is 69,7 kD:

Ila-Klon1 protein:	Molecular weight = 42,8 kD
GFP:	Molecular weight = 26,9 kD
Fusion protein:	Molecular weight = 69,7 kD

The BF-2 cells were examined daily for presence of fluorescence. In a great number of the BF-2 cells, fluorescence was clearly visible, mainly localised to cytoplasm.

Example 5. Expression in salmon

We want to examine the expression of the open reading frame protein in salmon as described for BF-2 cells in example 4. Partly, material from the immunised fish in example 6 may be used, but other application methods will also be applied. Following injection, samples from the site of injection will be removed, and following the use of for example gene gun, (Gene-gun, BioRad), it will be sufficient to scrape skin etc. Other possibilities will be taken to consideration accordingly. Performed tests will either be Western blotting or examination of fluorescence which detect presence of GFP.

Example 6. Immunisation of salmon

15 µg of the ILA-klon1-pEGFP-N1 plasmid disolved in 25 µl H_2O was injected intramuscularily in salmon with the size of 40-60 g. Insulin injection devise was used for the injection. A total number of 150 individuals were immunised. The fish were injected only once, i.e. no booster injection was administered

Example 7. Vaccine experiments followed by exposure to live ISA virus (Challenge)

In challenge experiments, individuals with a weight of 40-60 g were immunised with the ILA-klon1-pEGFP-N1 plasmid as described in example 6. Control individuals were immunised with the pEGFP-N1 plasmid devoid of insertion in the same amount and volume as the individuals given the ILA-klon1-pEGFP-N1 plasmid.

Following this immunisation, 4 weeks passed before the salmon was exposed to live ISA virus.

Exposure to ISA virus was performed by co-habitantly infection of the immunised salmons with ISA virus infected salmon.

Experimental ISA infection was performed by injecting salmon with the weight of 40-60 g intraperitoneally with ISA virus from cell culture supernatant diluted 1:10 in cell culture medium. The virus strain was Glesvær. Virus titre was 10^4/mL. Injection dosage: 0,3 mL intraperitoneally.

Subsequently, salmon with different immunisation/infection was distributed in the following way:

Tank A: 25 fish vaccinated with Klon1-pEGFP-N1
25 fish vaccinated with pEGFP-N1
5 experimentally ISA virus infected fish
introduced after 4 weeks

Tank B: 25 fish vaccinated with Klon1-pEGFP-N1
25 fish vaccinated with pEGFP
5 experimentally ISA virus infected fish
introduced after 4 weeks

Tank C: 100 fish vaccinated with Klon1-pEGFP-N1
5 experimentally ISA virus infected fish
introduced after 4 weeks

Tank D: 100 fish vaccinated with pEGFP-N1
5 experimentally ISA virus infected fish
introduced after 4 weeks

Thereafter, status between dead and surviving fish is made after 4-6-8 weeks.

Example 8. Vaccine effect in challenge experiments.

It is expected that degrees of protection against ISA infection may be detected. Any kind of protection will be regarded as a positive result. In the experiment, the fish is exposed to a very high transmission pressure, probably higher compared to natural exposure. Over a period of time, one cannot expect the fish to be protected.

Therefore, delay of clinical signs of disease compared to control fish will also be regarded as a positive result.

The results of the challenge tests gave no significant difference between vaccinated and control groups.

Example 9. Vaccine experiments using inter alia scaled infection pressure.

Subsequently to example 6-8, new vaccine experiments will be performed. The experiments will be carried out likewise as mentioned, but here with optimalisation regarding either/or application methods or/and type and amount of infection pressure.

Regarding applications, intracutaneous injection using gene gun (BioRad) will be valuated together with bath/dip. Regarding infection amount, one wish primarily to reduce the infection pressure in various ways, for example by reducing amount of experimentally infected fish or by reducing exposure period in the experiment.

Example 10. Vaccine experiment repeated as in examples 6-9 with some modifications.

The fish were somewhat smaller than 25 g and were injected with 25 µg of the ILA-klon1-pEGFP-N1 plasmid diluted in 100 µl TE with 2% polyvinylpyrrolidon-40. The fish were immunised twice with a 25 days interval. Only the experiments in tank A and B from example 7 were carried out. In tank A, the fish died rapidly, and there was not detected any difference between the groups. On the contrary, in tank B, the mortality was delayed. This may indicate a delayed infection impact compared to the expected 40 days. The experiment was terminated after ca. 17 weeks. In tank B, a significant difference between the groups was registered, this means that there were more survivors within the vaccinated groups. Using the X^2 test, a value of 5.36 was estimated which was significant at $p<0.025$. The values used in the estimations originated from the termination of the experiment, at which point 24 and 18 individuals in the groups of 25 were dead, in which 18 originated from the groups of vaccinated fish.

These results may indicate that a prolonged period of time between vaccination and exposure would have been better experimentally because protection against infection was somewhat more delayed than expected. In other words, the immune system of the fish needs somewhat longer time following DNA vaccination to achieve a good protective response.

Example 11. Identification of ISA virus

Organ material from ISA infected and non-infected salmon was homogenised, and a RNA extraction was carried out in accordance with commonly known biotechnological methods. Subsequently, Ready-To and RT-PCR Bead (Pharmacia) in the RT-PCR procedure was used. The reverse transcription and PCR reactions were performed as described by the manufacturer. PCR cycle using ISA primers is 95^0C 30 s, 55^0C 15 s and 72^0C 30 s, 35 cycles totally. Primers reacting with ISA virus sequences in accordance to claim 1-6 (labelled 1A and 1B) were used and compared to primers (labelled ILA1 and ILA2) reacting with ISA virus sequences and which are used for diagnostic purposes today. To visualise the reaction, a 3% NuSieve agarose in 1 x TAE buffer was used for electrophoresis at 80 volts in 75 minutes, a 123 bp ladder was used as size standard. The gel was put on an UV table and photo was taken.

35 samples from Tha Norwegian School of Veterinary Science (NSVS) and 54 samples from Scotland were tested. All samples from NSVS tested positively with respect to presence of ISA virus, and the Scottish samples tested negatively. The results show that the sequences mentioned in claim 1-6 with fitting primers are well suited for diagnostic purposes.

Sequence list

SEQ ID. NO.:1

The actual sequence corresponds to assumed gene segment 7 in the ISA virus.

Total length is 1320 nucleotides.

The sequence in this gene segment printed in the 5' to 3' direction is:

```
   1  AGUAAAAAAU GCACUUUUCU GUAAACGUAC AACAUCAAGA ACGUCUUCAA
  51  CCAAAAACAA UUUUACAUAC AUCUUUAAAA UUAAAAUCAC AUUAGAAUAC
 101  ACUCAUUAAC UUUUAACAUA GUUGCUUUUC UUUCAUAAUU AAGCAACAGA
 151  CAGGCUCGAU GGUGGAAUUC UACCUCUAGA CUUGUACAUG AAUGCUGCAA
 201  UCCAAAUACA UGCUUUCCAA CCUGCUAGGA ACAGAGCAAU CCCAAAACCU
 251  GCUACACCCA UAGUUUGGUU CAGCUGAGGU GGGAUUGCGU CUACCCUGAU
 301  CUUUACAUCU GUGAUGUUCC UCUGAAGUUU ACUGAUCAUC UCCUUAUGCA
 351  AUGCUACCUC CCUCAUGAUA AGUGUGUCUG UGUUACCUAA GCUUGUCUGG
 401  UCAAAAUCUU UAACCAUCUU AGGGCAGCUG UCAUAUUCAC ACGAAGCAGC
 451  AUCAAUUCUA CUCCAGUCAA UGUCUGGGCA UGUCAAUGCA UCAGUGAGGA
 501  ACGUUCUAAC GGAAGCUGAA CCAUGUAGUG AGUCAAAACC AUCAGAAGGA
 551  UAAACACCCC CUGUAUUUGG UGUGUCUACA UACUCAUCUA GCUCAACGUU
 601  CCUCAUGAUG UGUGAACACA AAGGCGUAUC ACUAGAAGAU CUUAGCAGAA
 651  CACACUUGUU UUUGGUUCCC CUCACUUCAA AGGUGUCUGA CACGUAGAUU
 701  UGUCCUUGGA AAUUGUCAAA CCUCAAAGGU GUGUUGAAAU ACCCGCAUCC
 751  GUUGAUCAGU UUCACUCCUC CCACAUUCCC AGAUGUUGCU GCAAUGCAGA
 801  CCUUGUAGAU UCCGGACAUU CCAUUCAAUC CAACCACUAC AAUUGUAGGG
 851  CUGCUGAAAG UCAACACCUU CACCGAAAAA CCGGUAACUG CGUCUGUUCG
 901  UCCAACAAGU CCAACAAUUG CUGCUCCCAC AAAUGUAGGC GUCACUCUCA
 951  CGUCUCCCCU GCAUGCUCCC AGGUACAGCU CCCUUGCCGC GUUGUCCAGU
1001  GUCAUCGAAG UUGGGAUGAU CAUGUCAGUA GGUCCGUCAG UGCAGUCAUU
1051  GGUUCCCAAG UAUAGGAUCC GGUACUUUGU AGUAGGUGUG UACCAGUCAC
1101  UUGGAAACCU UCCACUCAUC UGCUUCAAGA GUCCAUUUCC GUUUUUGGCC
1151  UGCAGCACCC CCUUGAACUC AGUCACCAGA UCCAAAGACU GUGGAUUCAC
1201  UCUAGACUGA UCGCUUCGAG AGUCACCUAU CCAGGUGGUG UCAGGGUAGU
1251  UUCUAAGACA UAGACGACUG UAAACAGGCG CCAACAGUAG GAAUAAAAUU
1301  AUGAAUCGUG CCAUCUUUGC
```

Originally, the gene segment was cloned as mRNA.

Naturally, occurrence of this gene segment is as RNA with negative sense, that is opposite sense of mRNA which per definition is positively sensed. The listed sequence encodes therefore not per se virus proteins, but it has to be transcribed to positive sense initially.

The sequence to positive sense, and re-written to DNA (U to T) is:

```
   1  GCAAAGATGG CACGATTCAT AATTTTATTC CTACTGTTGG CGCCTGTTTA
  51  CAGTCGTCTA TGTCTTAGAA ACTACCCTGA CACCACCTGG ATAGGTGACT
 101  CTCGAAGCGA TCAGTCTAGA GTGAATCCAC AGTCTTTGGA TCTGGTGACT
 151  GAGTTCAAGG GGGTGCTGCA GGCCAAAAAC GGAAATGGAC TCTTGAAGCA
 201  GATGAGTGGA AGGTTTCCAA GTGACTGGTA CACACCTACT ACAAAGTACC
 251  GGATCCTATA CTTGGGAACC AATGACTGCA CTGACGGACC TACTGACATG
 301  ATCATCCCAA CTTCGATGAC ACTGGACAAC GCGGCAAGGG AGCTGTACCT
 351  GGGAGCATGC AGGGGAGACG TGAGAGTGAC GCCTACATTT GTGGGAGCAG
 401  CAATTGTTGG ACTTGTTGGA CGAACAGACG CAGTTACCGG TTTTTCGGTG
 451  AAGGTGTTGA CTTTCAGCAG CCCTACAATT GTAGTGGTTG GATTGAATGG
 501  AATGTCCGGA ATCTACAAGG TCTGCATTGC AGCAACATCT GGGAATGTGG
 551  GAGGAGTGAA ACTGATCAAC GGATGCGGGT ATTTCAACAC ACCTTTGAGG
 601  TTTGACAATT TCCAAGGACA AATCTACGTG TCAGACACCT TTGAAGTGAG
 651  GGGAACCAAA AACAAGTGTG TTCTGCTAAG ATCTTCTAGT GATACGCCTT
 701  TGTGTTCACA CATCATGAGG AACGTTGAGC TAGATGAGTA TGTAGACACA
 751  CCAAATACAG GGGGTGTTTA TCCTTCTGAT GGTTTTGACT CACTACATGG
 801  TTCAGCTTCC GTTAGAACGT TCCTCACTGA TGCATTGACA TGCCCAGACA
 851  TTGACTGGAG TAGAATTGAT GCTGCTTCGT GTGAATATGA CAGCTGCCCT
 901  AAGATGGTTA AAGATTTTGA CCAGACAAGC TTAGGTAACA CAGACACACT
 951  TATCATGAGG GAGGTAGCAT TGCATAAGGA GATGATCAGT AAACTTCAGA
1001  GGAACATCAC AGATGTAAAG ATCAGGGTAG ACGCAATCCC ACCTCAGCTG
1051  AACCAAACTA TGGGTGTAGC AGGTTTTGGG ATTGCTCTGT TCCTAGCAGG
1101  TTGGAAAGCA TGTATTTGGA TTGCAGCATT CATGTACAAG TCTAGAGGTA
1151  GAATTCCACC ATCGAGCCTG TCTGTTGCTT AATTATGAAA GAAAAGCAAC
1201  TATGTTAAAA GTTAATGAGT GTATTCTAAT GTGATTTTAA TTTTAAAGAT
1251  GTATGTAAAA TTGTTTTTGG TTGAAGACGT TCTTGATGTT GTACGTTTAC
1301  AGAAAAGTGC ATTTTTTACT
```

The distribution of nucleotides in this sequence is:

A: 375 (28.4%) C: 249 (18.8%) G: 332 (25.2%) T: 364 (27.6%)

This sequence encodes following expected proteins:

Start is nucleotide 7, stop after nucleotide 1183.

```
  1  MARFIILFLL LAPVYSRLCL RNYPDTTWIG DSRSDQSRVN PQSLDLVTEF
 51  KGVLQAKNGN GLLKQMSGRF PSDWYTPTTK YRILYLGTND CTDGPTDMII
101  PTSMTLDNAA RELYLGACRG DVRVTPTFVG AAIVGLVGRT DAVTGFSVKV
151  LTFSSPTIVV VGLNGMSGIY KVCIAATSGN VGGVKLINGC GYFNTPLRFD
201  NFQGQIYVSD TFEVRGTKNK CVLLRSSSDT PLCSHIMRNV ELDEYVDTPN
251  TGGVYPSDGF DSLHGSASVR TFLTDALTCP DIDWSRIDAA SCEYDSCPKM
301  VKDFDQTSLG NTDTLIMREV ALHKEMISKL QRNITDVKIR VDAIPPQLNQ
351  TMGVAGFGIA LFLAGWKACI WIAAFMYKSR GRIPPSSLSV A*
```

Molecular weight = 42755.68 Residues = 391

Average Residue Weight = 109.350 Charged = 2

Isoelectric point = 7.66

Extinction coefficient = 45750

Residue	Number	Mole Percent
A = Ala	24	6.138
B = Asx	0	0.000
C = Cys	11	2.813
D = Asp	28	7.161
E = Glu	8	2.046
F = Phe	17	4.348
G = Gly	33	8.440
H = His	3	0.767
I = Ile	24	6.138
K = Lys	16	4.092
L = Leu	36	9.207
M = Met	11	2.813
N = Asn	17	4.348
P = Pro	19	4.859
Q = Gln	10	2.558
R = Arg	22	5.627

S = Ser	31	7.928
T = Thr	32	8.184
V = Val	31	7.928
W = Trp	5	1.279
Y = Tyr	13	3.325
Z = Glx	0	0.000
A + G	57	14.578
S + T	63	16.113
D + E	36	9.207
D + E + N + Q	63	16.113
H + K + R	41	10.486
D + E + H + K + R	77	19.693
I + L + M + V	102	26.087
F + W + Y	35	8.951

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Patent claims

1. DNA sequences to be used for vaccine purposes, profylactic health care in fish and aquatic organisms, including diagnostic systems, and in bio medicine as such, especially including human medicine and generally within research, as for example model organisms for influenza-like virus, which DNA sequences are characterised by encoding at least one protein from ISA virus, represented by the nucleotide sequence shown in SEQ ID.NO.:1 or sequences with at least 80 % homology to this.

2. DNA sequence according to claim 1, which is characterised by including parts of the nucleotide sequence shown in SEQ ID.NO.:1.

3. DNA sequences according to claim 1, which are characterised by having at least 80% homology/identity to the sequences according to claims 1-2.

4. Vector, which is characterised by containing DNA sequences according to claims 1-3.

5. Vaccine against ISA virus, which is characterised by including DNA sequences according to claim 1, which encode for at least one protein from the virus.

6. Vaccine against ISA virus, which is characterised by encoding a DNA sequence according to claim 1, which includes the nucleotide sequence shown in SEQ ID.NO.:1, or sequences with at least 80 % homology to this .

7. Vaccine against ISA virus, which is characterised by including a DNA sequence according to claim 2, which includes parts of the nucleotide sequence shown in SEQ ID.NO.:1.

8. Vaccine against ISA virus, which is characterised by including sequences according to claim 6, which have at least 80 % homology/identity with the sequences described in claim 1.

9. Diagnostic kit, which is characterised by comprising primers reacting with ISA virus sequences according to claims 1-3, for the detection of ISA specific nucleic acids or proteins.

Figure 1

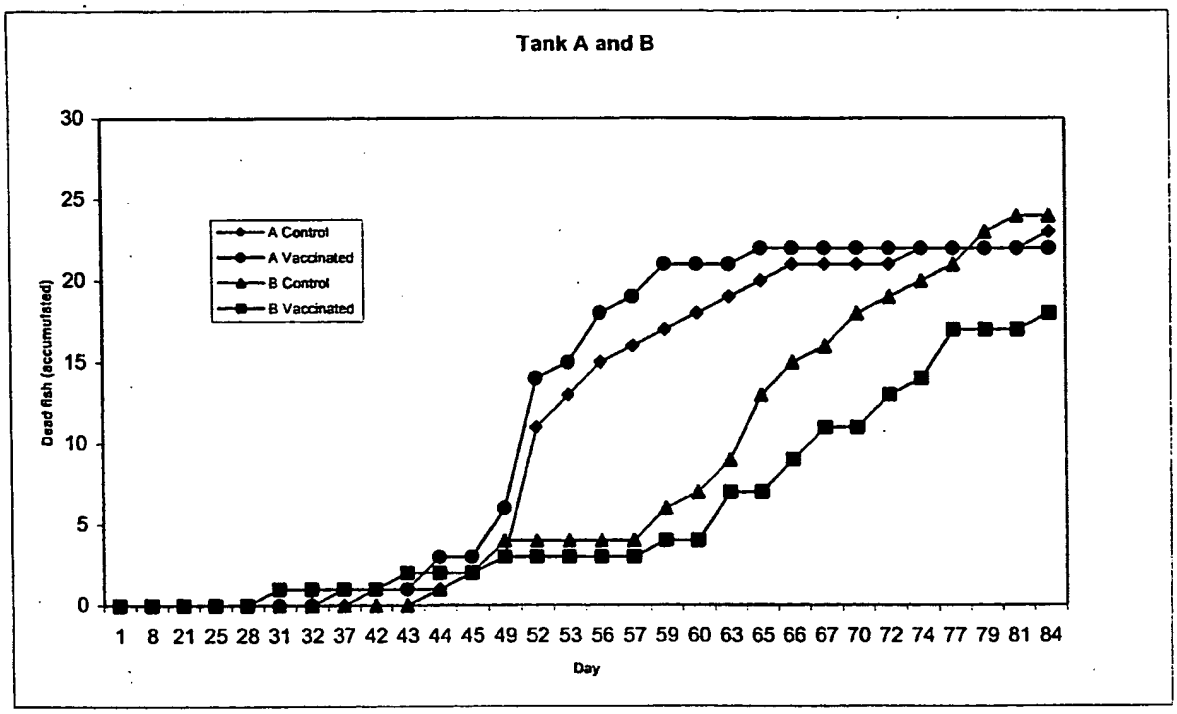
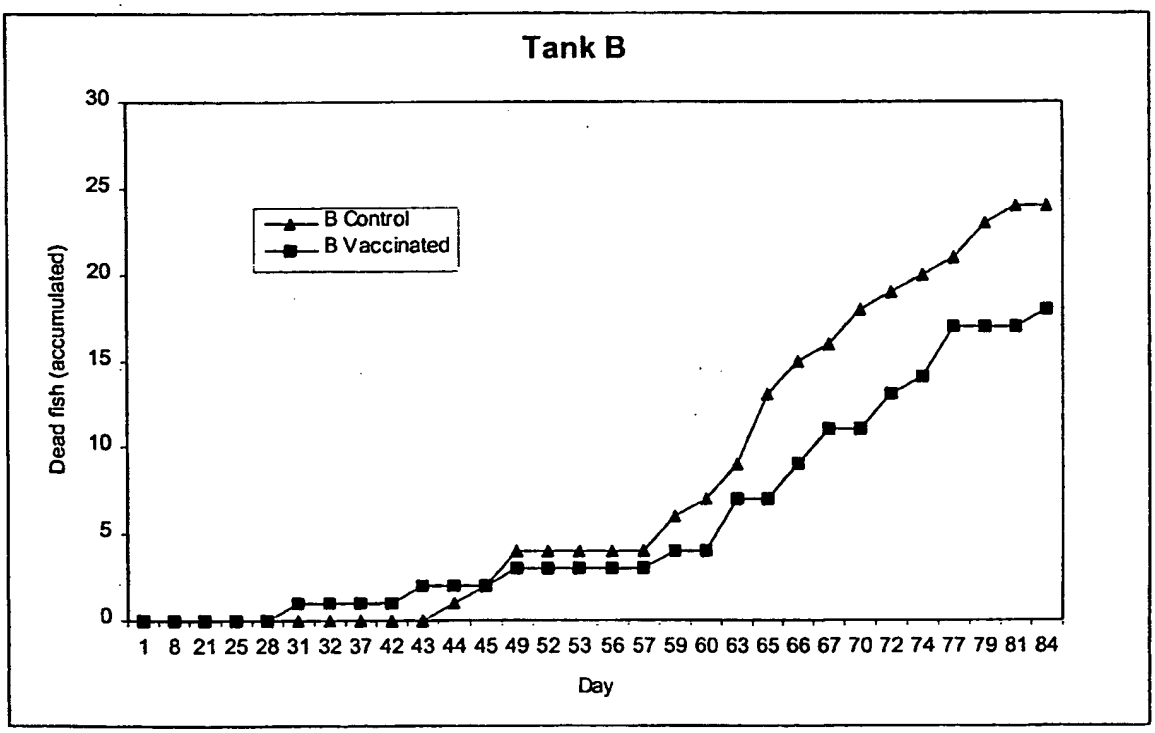


Figure 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 00/00179

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 39/145, C07K 14/11
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF VIROLOGY, Volume 71, No 10, October 1997, S. MJAALAND ET AL, "Genomic Characterization of the Virus Causing Infectious Salmon Anemia in Atlantic Salmon (Salmo salar L.): an Orthomyxo-Like Virus in a Teleost" page 7681 - page 7686	1-4,9
Y	--	5-8
X	Journal of Virology, Volume 73, No 3, March 1999, Bjørn Krøssoy et al, "The Putative Polymerase Sequence of Infectious Salmon Anemia Virus Suggests a New Genus within the Orthomyxoviridae" page 2136 - page 2142	1-4,9
Y	--	5-8

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
18 Sept 2000	20 -09- 2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson/GH Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	National Library of Medicine (NLM), file Medline, Medline accession no. 99192938, Lovely JE et al: "First identification of infectious salmon anaemia virus in North America with haemorrhagic kidney syndrome."; & Dis Aquat Organ 1999 Jan 29;35(2): 145-8	1-4
Y	--	5-9
Y	US 5780448 A (HEATHER L. DAVIS), 14 July 1998 (14.07.98)	5-8
	--	
Y	EP 08399136 A2 (OTTAWA CIVIC HOSPITIAL), 6 May 1998 (06.05.98)	5-8
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X	JOURNAL OF VIROLOGY, Volume 71, No 12, December 1997, KNUT FALK et al, "Characterization of Infectious Salmon Anemia Virus, an Orthomyxo-Like Virus Isolated from Atlantic Salmon (Salmo salar L.)", page 9016 - page 9023, see page 9017, left column	9
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NO 00/00179

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5780448 A	14/07/98	CA 2189831 A	08/05/97
		EP 0773295 A	14/05/97
		JP 9285291 A	04/11/97
		NO 964713 A	09/05/97
		EP 0839913 A	06/05/98
EP 08399136 A2	06/05/98	NONE	

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